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Laboratory Evaluation of the Efficacy of 10% Imidacloprid + 2.5% Moxidectin Topical Solution (Advantage® Multi, Advocate®) for the Treatment of *Dirofilaria immitis* Circulating Microfilariae in Dogs

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Abstract

This study examined the efficacy of 10% imidacloprid + 2.5% moxidectin topical solution (Advantage® Multi, Advocate®, Bayer) for the treatment of circulating microfilariae from dogs naturally infected with *Dirofilaria immitis*. The study included two groups of 11 dogs each that consisted of two replicates. Replicate 1 contained 12 dogs (6 treated and 6 controls) and replicate 2 contained 10 dogs (5 treated and 5 controls). Six of the 10 dogs in replicate 2 were the controls from replicate 1. All dogs entering the study completed a physical examination including chest radiographs, blood collections for examination of *Dirofilaria immitis* circulating microfilariae, serum chemistry, complete blood counts and urinalysis. To qualify for the study each dog was required to have a geometric mean ≥300 microfilariae per ml of blood from 3 consecutive samples collected during the 8 day acclimation period and a heartworm disease classification of 1 or 2. Dogs were treated on study days 0 and 28. Post-treatment microfilarial counts were performed on study days 1, 2, 3, 7, 14, 21, 28, 29, 35, and 42. Percent microfilarial reduction was determined by comparing the geometric mean number of circulating microfilaria remaining in treated dogs with those remaining in the control dogs post-treatment. Seven days after the first treatment, the geometric mean microfilarial counts in treated dogs were reduced by >99% compared to the control dogs. Reduction remained at >99% through the end of the study at 42 days after the first treatment (14 days after the second treatment). The results of this study demonstrated that *Advantage® Multi for dogs* is efficacious for treatment of circulating *D. immitis* microfilariae in naturally infected heartworm-positive dogs with no treatment-related adverse events observed.

Introduction

The removal of *Dirofilaria immitis* circulating microfilariae in conjunction with or following the administration of an adulticide is of paramount importance for improving the clinical condition of the dog and mitigating the spread of heartworm infection (McCall et al 2014). Topically applied *Advantage® Multi for Dogs* received additional label claims for "the treatment of *Dirofilaria immitis* circulating microfilariae in heartworm-positive dogs and the treatment and control of sarcoptic mange caused by *Sarcoptes scabiei* var. *canis*" (Supplemental NADA 141–251 approved October 24, 2013). These claims were added to the original approved claims for killing adult fleas and the treatment and control of nematode infections, including fourth-stage larvae, immature adults, and adult stages of *Ancylostoma caninum* and *Uncinaria stenocephala*, fourth-stage larvae and adult *Toxocara canis* and adult *Toxascaris leonina* and *Trichuris vulpis*. The label also includes monthly use for preventing the development of canine heartworm disease caused by *Dirofilaria immitis* (Arther et al. 2005). The supporting data for this product for the treatment of dogs for microfilariae following transplantation of adult *D. immitis* (Pepper strain, TRS Labs, Inc, Athens, GA) has previously been reported (McCall et al. 2014). In addition, a clinical field study for further safety and efficacy evaluation was performed at clinical sites in Alabama, Louisiana, Missouri, Oklahoma, Tennessee, and Texas where the product was used alone or in conjunction with melarsomine dihydrochloride for removal of adult worms (Supplemental NADA 141–251 approved October 24, 2013). The objective of the study reported here was to evaluate the efficacy of *Advantage® Multi for Dogs* for the treatment of circulating *D. immitis* microfilariae at the minimal labelled dose of 0.1 ml per kilogram body weight for naturally heartworm infected dogs held under laboratory conditions.

Materials and Methods

Animals

Twenty-two (22) dogs obtained from different USDA licensed (U.S. Department of Agriculture (USDA) Animal Welfare Act (9 CFR Parts 1, 2, and 3) sources, that were demonstrated to have adequate pre-treatment mean circulating *D. immitis* microfilariae (mff) counts of ≥ 300 mff per ml of blood, were included in the study and were randomised to two groups of 11 animals per group. Animals were acclimatised at the facility at least 8 days prior to enrollment. General health observations were conducted once daily. During the acclimatisation period, urinalysis was performed and blood was collected for serum chemistry, CBC (complete blood count), and for quantification of circulating mff using the modified Knott's test (Bowman 2009). In addition, pre-treatment chest radiographs were performed and body weights were collected. These tests and examinations were used to determine the heartworm disease classification for each dog. Only dogs with a heartworm disease Classification of 1 or 2 were included in the study (American Heartworm Society 2012; Louisiana State University School of Veterinary Medicine 2015).

The dogs were housed in raised stainless steel pens in climate controlled rooms. The space allocation for each animal was in accordance with pertinent animal welfare guidelines or regulations set forth by the USDA. All pens were enclosed on top. Polypropylene dividers prevented animal to animal contact and cross contamination between pens. Dogs were fed a daily ration of commercial dog food containing 21 % protein (River Run, Cargill Animal Nutrition, Minneapolis, MN) and provided water ad libitum. Pens were cleaned daily. Temperature and relative humidity were recorded once daily using maximum-minimum thermometers and a portable digital thermo-hygrometer. The temperatures and relative humidity were normal during the study. Each pen was identified with the animal ID, study number and gender. Lighting was

Table 1: Animal, body weight and treatment information of dogs used to determine the effects of treatment with either *Advantage® Multi for Dogs* or mineral oil on study days 0 and 28.

Treatment Group	Dog ID*	Sex	Day 1 Body Weight (kg)	Day 0 Dose (ml)	Day 27 Body Weight (kg)	Day 28 Dose (ml)
Advantage® Multi for dogs	13584–1	F	12.70	1.27	12.05	1.21
	13697–2	M	26.75	2.68	26.95	2.70
	13734–2	M	26.35	2.64	25.80	NA**
	13777–2	F	31.25	3.13	30.0	3.00
	13778–1	M	43.75	4.38	43.05	4.31
	2167–1	F	31.00	3.10	30.75	3.08
	2759–2	M	40.30	4.03	41.55	4.16
	E7934–1	M	29.50	2.95	29.65	2.97
	E7983–2	F	31.90	3.19	31.15	3.12
	E7987–1	F	26.90	2.69	24.90	2.49
	E7988–1	F	31.85	3.19	31.15	3.12
Mineral Oil	13680–1	F	24.95	2.50	24.55	2.46
	13680–2	F	25.15	2.52	24.10	2.41
	13697–1	M	28.20	2.82	27.00	2.70
	13734–1	M	26.70	2.67	26.55	2.66
	13792–2	M	28.70	2.87	26.80	2.68
	2224–1	F	17.55	1.76	17.65	1.77
	2224–2	F	17.35	1.74	17.75	1.78
	2755–2	F	22.75	2.28	21.70	2.17
	58101–1	M	20.45	2.05	23.45	2.35
	58101–2	M	22.50	2.25	21.80	2.18
	E7983–1	F	31.60	3.16	30.20	3.02

*ID + replicate number
** Treatment not administered

provided by overhead fluorescent lamps, and an automatic timer provided approximately 12 hours of light and 12 hours of darkness each day. General health observations were conducted daily beginning on studay day (SD) -8. The animals were maintained with due regard for their welfare and in accordance with applicable laws, regulations and guidelines. The protocol was approved by the Institutional Care and Use Committee prior to initiation of the study. The study was conducted in accordance with the principles of FDA Guidance for Industry 85, Good Clinical Practice, VICH GL9, May 2001.

Study Design

This study was conducted utilising two replicates. Replicate 1 included 12 dogs. Replicate 2 included 10 dogs (6 of these were dogs from the control group utilised from the completed Replicate 1) (Table 1). All dogs received a physical examination and were weighed during acclimatisation on SD -7 in Replicate 1 and SD -8 in Replicate 2. Animals were randomised on SD -1, by mean pre-treatment mff counts to one of two study groups. Three pre-treatment mff counts were performed for each dog using the modified Knott's test on

Table 2: Microfilarial counts per milliliter of blood of dogs treated with *Advantage® Multi for dogs* or mineral oil before and after treatment. The first treatment occurred on Day 0 and a second treatment occurred on Day 28.

Dog-Replicate	Day Pre or Post Treament															
	-8	-7	-6	-5	-4	Mean Pre-Treatment	1	2	3	7	14	21	28	29	35	42
	Microfilariae per Milliliter of Blood															
Advantage® Multi for dogs																
13584–1	*	*	4400	9000	3250	5550	12800	9850	6900	65	4	0	0	0	0	0
13697–2	7400	1650	6900	*	*	5317	6750	9850	6500	30	4	2	12	*	3	0
13734–2	2650	1800	2650	*	*	2367	3150	400	57	35	9	8	9	*	*	*
13777–2	16650	28800	42950	*	*	29467	24300	3600	750	105	42	40	29	*	14	0
13778–1	*	*	1000	1550	750	1100	1200	600	200	0	0	0	0	0	0	0
2167–1	*	*	2250	1800	11650	5233	2050	3200	3200	75	0	4	14	9	6	17
2759–2	8650	5650	6750	*	*	7017	2950	100	54	1	0	0	0	*	0	0
E7934–1	*	*	10650	7000	12700	10117	8450	4000	3000	27	0	0	0	1	0	0
E7983–2	8550	2700	4650	*	*	5300	15050	6950	7700	648	75	17	30	*	19	0
E7987–1	*	*	550	1900	600	1017	200	450	350	0	0	0	0	0	0	0
E7988–1	*	*	1350	2750	41000	15033	1050	1050	1450	9	0	0	0	0	0	0
Placebo																
13680–1	*	*	15700	9600	15900	13733	18050	28550	10600	14800	10200	22950	18250	11700	37750	10800
13680–2	27900	18900	5550	*	*	17450	32850	36900	36450	33600	42750	34200	27450	*	28550	27450
13697–1	*	*	9250	17400	3450	10033	7250	5250	5450	6200	5850	5600	9250	4700	4400	9450
13734–1	*	*	1100	1900	5850	2950	1200	4500	850	4000	1000	2750	1500	3050	1150	1500
13792–2	31500	31050	38200	*	*	33583	62100	27000	23450	16200	36450	22950	23400	*	18550	21150
2224–1	*	*	1700	3500	25900	10367	2800	7450	5900	3450	1950	3100	1450	750	2500	11700
2224–2	1900	3950	3800	*	*	3217	4250	3900	5600	4000	3900	5400	6000	*	6850	5800
2755–2	1400	7250	8700	*	*	5783	9300	10300	2300	7100	3550	4950	5350	*	4900	2000
58101–1	*	*	500	650	600	583	500	500	500	200	200	250	200	450	550	300
58101–2	350	450	700	*	*	500	350	250	700	600	700	550	600	*	450	250
E7983–1	*	*	900	10800	1100	4267	1250	2150	1400	350	1250	1750	7300	5400	5350	3550

* Microfilarial counts were not performed on these days from these dogs.

SDs -6, -5 and -4 (Replicate 1) and -8, -7 and -6 (Replicate 2) (Table 2). Dogs with pre-treatment mean counts of at least 300 mff per mL that also met the other inclusion criteria were ranked highest to lowest by their mean pre-treatment mff counts. The first 2 dogs (highest counts) were assigned to Set 1, the next 2 dogs were assigned to Set 2, and so forth, until the final 2 dogs (lowest counts) were assigned to Set 6 for Replicate 1. This same allocation method was utilised for Replicate 2, Sets 1 through 5.

Within the two replicates, animals in Group 1 (treated dogs) were treated twice (once on SD 0 and once on SD 28) with *Advantage® Multi for dogs* at the minimum label dose (0.1 ml per kilogram body

weight), and Group 2 animals (control dogs) in each replicate were treated twice (once on SD 0 and once on SD 28) with mineral oil of an equivalent volume to the volume of *Advantage® Multi for Dogs* to serve as a negative control to mask the treatment received by the two different groups. Animals were reweighed on SD 27, prior to the second treatment (SD 28) (Table 1).

The dogs were observed at 1, 2, 3, 4, 5, 6, 8, 12 and 24 hours post-treatments on the days of treatment on SDs 0 and 28. The dogs were observed once daily on all other post-treatment study days. Study personnel responsible for post-treatment safety observations and efficacy assessments were masked to treatment designations. Blood samples for mff counts were collected on SDs 1, 2, 3, 7, 14, 21, 28, 29, 35, and 42.

Microfilarial counts

Microfilariae were counted using the modified Knott's test (Bowman 2009). Briefly after the Knott's sedimentation was performed, the volume in the tube was adjusted to 1 ml with 2% formalin. Then 20 µl of the mixed 1 ml was examined for microfilariae, and, if mff were found, the count was multiplied by 50 to give the number per ml. If no mff were observed, an additional 100 µl was examined, and, if mff were found, the count was multiplied by 8.33 to determine the number of mff per ml. If no microfilariae were seen in the first 120 µl volume, the entire sediment was examined with the total number of mff observed considered to be the number of mff per ml.

Data Analysis

Percent mff reduction was determined by comparing the geometric mean number of circulating mff remaining in Group 1 with the mff counts remaining in Group 2 at post-treatment intervals on SDs 1, 2, 3, 7, 14, 21, 28, 35, and 42. The graph with mean and 95% confidence intervals in Figure 1 was created by Prism version 6.04, January 17, 2014, GraphPad Software, Inc.

The analysis consisted of a hierarchical assessment, using the microfilarial counts made on samples collected on SDs 28 and 42. The assumption was, that the counts collected from samples on the treated animals on these two days would have 90% or greater reduction of the mff compared to the animals in the control group. For the statistical comparisons, the microfilarial counts were transformed [log(counts+1)] and analysed with an analysis of covariance (ANCOVA) including terms for treatment (TRT) (fixed), and block and animal (random), using the average of the log(pre-treatment count+1) values as a covariate. SAS PROC MIXED (SAS® version 9.2, SAS® Institute, Cary, NC) was used. The TRT main effect was evaluated. Comparisons were made between the treatment groups using LSMEANS, ($p \leq 0.05$, two-sided).

To provide substantial evidence of the effectiveness of topically applied *Advantage® Multi for Dogs* in a laboratory setting, a mff percent reduction of 90% or greater and a statistically significant difference between the control and treated groups was required at SD 42 and/or 28, and at least 6 of the animals in the control group were required to have an adequate infection of ≥300 mff/mL on SD 42 and/or 28.

The efficacy evaluation was based on the percent reduction of the geometric mean mf counts for *Advantage® Multi for Dogs* treated dogs when compared to the control dogs topically treated with mineral oil for each post-treatment mff count. Percent efficacy (or percent mff reduction) was determined by comparing the geometric mean mff counts recorded for the IVP treated groups to the geometric mean mff counts recorded for the control group using Abbott's formula:

% Efficacy = (N1–N2)/N1 x 100
N1: Geo. mean mff count $_{(Control)}$ N2: Geo. mean mff count $_{(Treated)}$

Results

Microfilariae counts for the three blood samples collected prior to the first treatment ranged from 500 to 33,583 mff per ml (Table 2). In the control dogs, at 3 days after the mineral oil application, the mff counts ranged from 500 to 36450 mff per ml, while the counts in the treated dogs ranged from 54 to 7,700 mff per ml. A week after the first treatment, the counts in the control dogs ranged from 200 to 33,600 mff per ml while the counts in the *Advantage® Multi for dogs* treated dogs decreased to 0 to 648 mff per ml. By the day of the second treatment on SD 28, the counts in the control dogs ranged from 200 to 27,500 mff per ml, while in the treated dogs the counts ranged between 0 to 30 mff per ml with 5 of the 11 dogs having microfilariae. On SD 42, the last day of sampling, only one treated dog had microfilaria (17 mff per ml), while all control dogs remained positive with a range of 250 to 27,450 mff per ml. Based on the log10 conversion of the mff counts a 3 log reduction in circulating mff was achieved by SD 14 while the mff were almost totally absent on SD 42 in dogs treated with *Advantage® Multi for dogs* (Fig. 1).

The data demonstrate that mff counts in the treated dogs were statistically reduced as compared to the control dogs on SDs 28 and 42, with 10 of the 11 control dogs with mff counts > 300 mff per ml on Study Day 42. In this study, mff counts in the treated dogs were reduced by > 99 % by SD 7, following one topical treatment with *Advantage® Multi for dogs* compared to the control dogs that received mineral oil as a treatment with the microfilarial reduction remaining > 99 % through SD 42 (Table 3). The dogs treated with *Advantage® Multi for dogs* had significantly ($p < 0.05$) fewer microfilariae as compared to the control dogs on SDs 28 and 42.

No adverse events were observed which were considered to be treatment related.

Discussion

Advantage® Multi for Dogs is a unique combination of the insecticide imidacloprid and the endectocide moxidectin, that provides a broad spectrum of activity against internal and external parasites

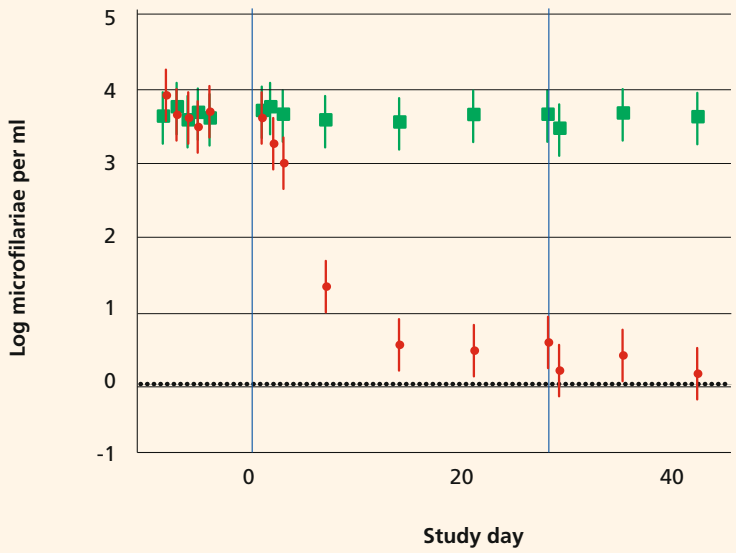

Fig. 1 Mean (and 95 % confidence interval) of log base 10 of microfilariae per milliliter of blood in two groups of 11 dogs. One group (red circles) was treated twice with *Advantage® Multi for dogs* and the other group was treated twice with a mineral oil placebo (green squares). The topical treatments were administered on Study Days 0 and 28; vertical lines at 0 and 28 on the x-axis represent the days the treatments were administered.

Table 3: Geometric Mean Microfilariae Counts and Percent Efficacy in dogs treated with *Advantage® Multi for dogs* or mineral oil. The first treatment occurred on Day 0 and a second treatment occurred on Day 28.

Study Day	*Advantage® Multi for dogs*	Placebo	Percent Efficacy*
-8	7526.4	3826.7	NA
-7	4199.1	5968.9	NA
-6	3728.2	352.7	NA
-5	3117.9	4454.0	NA
-4	4551.5	4200.7	NA
1	3593.1	4231.7	15.1
2	1706.2	5048.8	66.2
3	980.5	3657.8	73.2
7	19.1	3451.6	99.4
14	2.4	3260.0	99.9
21	1.8	4103.8	100.0
28	2.7	4172.4	99.9**
29	0.6	2596.3	100.0
35	1.5	4376.5	100.0**
42	0.3	3906.4	100.0

NA = Not applicable; * these numbers were rounded
** Log MF counts significantly different between two groups ($p < 0.05$) using separate analysis of covariance for each study day

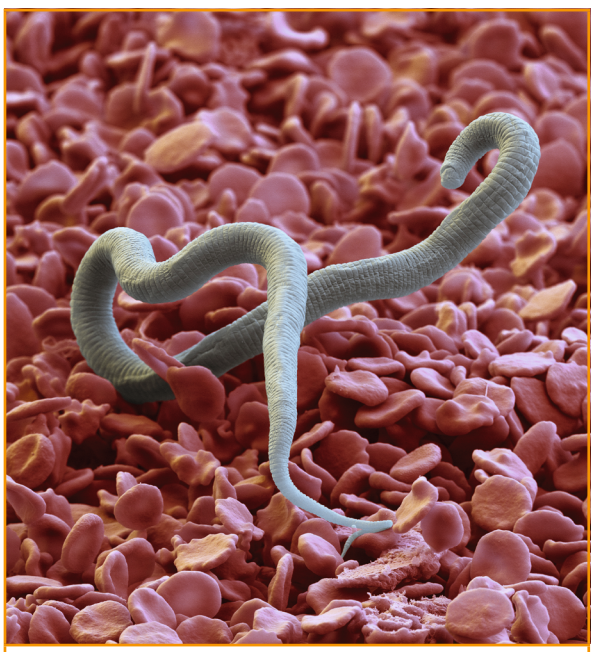

Fig. 2 Scanning electron micrograph of a microfilaria of Dirofilaria immitis

with the moxidectin component demonstrating high levels of efficacy against a range of stages and species of intestinal nematodes (Cruthers et al. 2008), including microfilariae of *D. immitis* (Hendrix et al. 1992). The effectiveness of 10 % imidacloprid / 2.5 % moxidectin in the treatment of dogs with microfilaria of *D. repens* has been demonstrated (Hellman et al. 2011; Traversa et al. 2011). Most preventive canine heartworm products are not given at dosages designed to be completely microfilaricidal (Bowman and Atkins 2009). It has been shown that, when moxidectin was given at a lower dosage (3 µg/kg body weight), it did not demonstrate marked microfilaricidal activity, but with a higher dosage showed an effect on mff after repeated administration (Hendrix et al 1992). Therefore, an increased dosage is necessary for macrocyclic lactones because microfilariae are typically less susceptible than immature (L3, L4) filariid larvae (Bowman 2012).

In this study, when dogs with circulating *D. immitis* mff were treated with *Advantage® Multi for Dogs* on SDs 0 and 28, microfilarial counts were reduced by > 99 % on SD 7 following a single treatment when compared to control dogs and remained at > 99 % through SD 42. The log of the mff counts were significantly lower in the *Advantage® Multi for Dogs* treated group on SDs 28 and 42 ($p < 0.05$) using separate analysis of covariance for each study day in comparison to counts on the control dogs. The results of this study demonstrated that 10 % imidacloprid + 2.5 % moxidectin topical solution (*Advantage® Multi for Dogs*) is efficacious for treatment of circulating *D. immitis* mff in heartworm-positive dogs with Class 1 or Class 2 heartworm disease presentation with no treatment-related adverse events observed.

Moxidectin is a highly lipophilic macrocyclic lactone, that is stored mainly in fat tissues (Al-Azzam et al. 2007) and achieves high plasma concentrations with gradual elimination from the host (Blagburn et al. 2009). Even though avermectins and milbemycins contain a common macrocyclic lactone ring, they display different ranges of potency, activity and pharmacokinetics (Prichard and Roulet 2005). Some avermectins and moxidectin have unique characteristics that allow for greater flexibility in use. The longer half-life and safety profile of moxidectin allows it to be used in long-acting formulations (Prichard et al. 2012), and interest has shifted on moxidectin as a filaricide (Geary and Mackenzie 2011). In a previous study, where dogs received adult heartworms by transplantation of 10 adult males and 10 adult females (Pepper strain, TRS Labs Inc., Athens, GA), mff counts for the *Advantage® Multi for Dogs* treated dogs were reduced 99.9 % following two treatments compared to the control group although not all treated dogs were totally cleared of mff on SD 42 (McCall et al. 2014).

Similarly, not all dogs were cleared of their mff in the clinical field study as detailed in the Freedom of Information Summary (Supplemental NADA 141–251, approved October 24, 2013). The field study included a total of 181 dogs from the six states used in the evaluation of *Advantage® Multi for Dogs* as a microfilaricide. Approximately 50 % of the dogs received the microfilaricidal treatment concomitant with adulticide therapy. The treatment regimen used a single injection of melarsomine dihydrochloride two weeks (SD -14) before the first *Advantage® Multi for Dogs* application (SD 0), and two additional melarsomine dihydrochloride injections two days in a row 2 weeks after the first *Advantage® Multi for Dogs* application (SDs 14 and 15), and the final *Advantage® Multi for Dogs* treatment 2 weeks after the melarsomine dihydrochloride treatments (SD 28). In the field study the mff counts for efficacy evaluation were made on SDs -14, 28, and 42. Even though not all the dogs in the field study were cleared of their microfilariae, the overall effectiveness of two treatments with *Advantage® Multi for Dogs* in reducing mff in dogs was very high, i.e., 99.3 % and 99.5 % on SDs 28 and 42, respectively.

It would be prudent of the veterinary community to verify that the mff are eliminated after treatment with *Advantage® Multi for Dogs,* in order to minimize the risk of these mff being transmitted to another dog via a mosquito. It may also be of interest to determine if there are cases where recrudescence of mff occurs, if treatment of a dog with adult worms is terminated after one or several apparently successful treatments with *Advantage® Multi for Dogs*.

Ethical Standards

The study was conducted in compliance with the VICH GL 9 Good Clinical Practice, Study Protocol and SOPs referred to in the protocol and with current applicable local and federal laws and regulations.

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Conflict of interest

Samuel D. Charles, Robert G. Arther, and Terry Settje are current employees of Bayer HealthCare. Dwight D. Bowman has no known conflicts of interest with this study.

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